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High Density of Tumor-infiltrating B-Lymphocytes and Plasma Cells Signifies Prolonged Overall Survival in Adenocarcinoma of the Esophagogastric Junction

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Abstract. *Background: Tumor-infiltrating lymphocytes (TILs) have been shown to be of prognostic significance in a variety of tumors. Not only T-cell, but also B-cell infiltration is commonly associated with improved survival. Materials and Methods: We assessed the density of tumor-infiltrating B-cells, as well as that of plasma cells, in 210 adenocarcinomas of the esophagogastric junction through immunohistochemical analysis using antibodies against CD20 and CD138. Results: No correlation between density of B-cells or plasma cells and various clinicopathologic features could be established. High density of tumor-infiltrating B-cells, as well as plasma cells, showed significantly better overall survival (OS) compared to patients with no infiltrates (p=0.047 and p=0.022, respectively). Cox proportional hazard analysis could verify B-cell infiltration as an independent prognostic factor (hazard ratio (HR)=0.683; 95% confidence interval (CI)=0.517-0.901; p=0.007). Conclusion: Plasma cell and B-cell infiltration correlates with prolonged OS and might identify a patient subset with favorable disease course.*

In recent years, studies concerning the microenvironment of tumors, especially tumor-infiltrating lymphocytes (TILs), have been abundant. This microenvironment has been shown to influence overall survival (OS), as well as relapse times, and probability of tumor recurrence (1, 2). However, most studies have focused on the relationship between T-cell infiltration and patients' outcome, while the role of tumor infiltrating B-lymphocytes (TIL-Bs), as well as plasma cells in this setting, is still poorly understood and less commonly investigated.

Not only the presence, but also the distribution pattern has been described as being crucial to prognosis: In carcinomas, immune cells are predominantly found in the stroma (3) and the formation of dense, follicle-like lymphocyte infiltrates (so-called tertiary lymphatic structures (TLSs), which arise in non-lymphoid tissues, often in the context of chronic inflammation) has been reported to have the strongest impact on prognosis and survival (2, 4, 5).

T-lymphocytes (especially cytotoxic T-cells), as well as B-lymphocytes, increase and establish the activation of antitumoral immunity (as an adaptive mechanism), leading to increased cell death in tumors and resulting in improved prognosis in patients with high levels of TILs (2, 6). Tumor-associated plasma cells have been described to have a significant impact on prognosis through modulation of the humoral immune response (7).

Up to date, most reports in the literature state a positive effect of TILs in respect to OS, relapse-free survival and association with favorable prognostic factors, as well as lower T-stage (8). High B-cell density is said to be associated with improved outcome in gastric, breast and colorectal cancer (9-11), as well as squamous cell carcinoma of the tongue (12), melanoma and hepatocellular carcinoma (5, 13). The same correlation has been shown for pancreatic adenocarcinomas, though here the impact was only measurable if cells were arranged in the pattern of TLSs (4, 14). High proportions of TILs have also been shown to be predictive of response to neoadjuvant therapy in breast cancer patients (15).

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However, there are also some studies showing that increased B-cell density is associated with higher recurrence frequencies in meningioma and prostate cancer, as well as with higher tumor grade and reduced OS in ovarian cancer or disease progression in breast cancer (16-19). Single studies state no prognostic effect of TIL-Bs (20).

Increased plasma cell density has been reported to be related to improved outcome in non-small cell lung cancer (NSCLC) and high-grade ovarian carcinoma (7, 21), while other studies showed an association with higher tumor grade and reduced OS in ovarian cancer (22).

The objective of this study was to analyze the potential prognostic value of tumor infiltrating immune cells in adenocarcinoma of the esophagogastric junction and their association with clinicopathological features.

Materials and Methods

Case selection. Two hundred and ten tumor samples (formalin-fixed, paraffin-embedded tissue (FFPE) of adenocarcinomas of the esophagogastric junction with their epicentre within 2 cm on either side of the junction) were collected as part of standard clinical care at the University Hospital Schleswig-Holstein, Campus Luebeck, Germany, during 1992 and 2014. The study was approved by the local Ethics Committee of the University of Luebeck (reference 14-242A).

Follow-up data was available for all patients with a mean follow-up period of 42.82 months (range=0-227).

Tissue microarray construction. Tissue microarrays (TMAs) were constructed using the manual QuickRay® kit (Unitma, Seoul, Korea) in accordance with the manufacturer's instructions. One representative core per tumor sample measuring 2 mm was dissected and mounted onto a tissue microarray. Appropriate positive and negative controls were included in each slide (kidney and tonsil tissue).

Immunohistochemistry. Immunohistochemical studies were performed on FFPE sections of the TMA according to a standard, three-step immunoperoxidase technique using the automated Menarini Bond Max System (Menarini Diagnostics, Berlin, Germany). The following antibodies were used: CD20 (clone L26, dilution 1:500; Cellmarque, Rocklin, CA, USA) and CD138 (clone B-A38, dilution 1:100; Biocare medical, Concord, CA, USA). All immunohistochemical stains were evaluated and a consensus was reached by two pathologists (JK, KR).

Scoring of cell density. Cell density was assessed semi-quantitatively with the following scoring system: 0 (no detectable B- or plasma cells); 1+ (scattered cells throughout the tumor); 2+ (cells organized in follicles and/or dense sheets, forming TLSs) as previously described (14). Representative examples of scoring system are shown in Figure 1.

Statistical analysis. Statistical analysis was conducted using SPSS Statistics version 22 (IBM, Ehningen, Germany). Survival differences and outcome, as well as OS were analyzed with Kaplan-Meier estimates, including log-rank test, while correlation of CD20 and CD138 expression with different clinicopathologic features (univariate analysis) was determined with χ^2 and Fisher's exact test, as well as Pearson's bivariate correlation. Cox regression analysis (proportional hazard ratio) was conducted to test for independence. Additionally, correlation between densities of cell infiltrates was evaluated by Spearman's Rho. A p-value <0.05 was considered statistically significant for all analyses.

Results

General aspects of the study group. The study population consisted of 210 individuals with 177 male (84.3%) and 33 female patients (15.7%). Ages ranged from 27 to 83 years with a mean age of 61.73 years (standard deviation±10.663). Fifty-nine patients (28.1%) received neoadjuvant therapy; for 67 patients (31.90%), no data regarding possibly administered neoadjuvant therapy was available.

Density of B-cell and plasma cell infiltrates. Overall, 108 tumors (51.4%) showed no plasma cell infiltrates, 60 cases (28.6%) only scattered cells (score 1+) and 36 tumors (17.1%) had dense infiltrates (score 2+). Concerning B-lymphocytes, 77 tumors (36.7%) showed no infiltration, 84 cases (40%) scattered (score 1+) and 48 tumors (22.9%) dense infiltrates (score 2+). Six tumors (2.9%) were not evaluable for plasma cell infiltration, 1 case (0.5%) was not evaluable for B-lymphocytes.

Correlation of density of B-lymphocytes and plasma cells. Analysis of correlation between cell infiltrates applying Spearman's Rho revealed only a weak association with a correlation coefficient of 0.312.

Correlation of B- and plasma cell density with clinical and pathologic features. Univariate analysis of immune cell infiltrates and various features of the study group (*i.e.* gender, pT-stage, nodal stage, grading, lymphovascular invasion, perineural invasion, presence of distant metastases and histologic subtype according to WHO classification) revealed no significant correlation with any of the mentioned features. Appropriate data including p-values are summarized in Table I.

Plasma cell density in tumor microenvironment. Concerning CD138 expression/density of plasma cells in the tumor microenvironment, there was a significant difference in survival between scores 0, 1+ and 2+ (p=0.045). Further analysis of patient groups with no plasma cell infiltration (score 0) against those with high density (score 2+) showed that patients' OS was significantly better in cases with high plasma cell density (p=0.022), as shown in Figure 2.

Survival times in scoring group 0 averaged 58.72 months (±8.308), 70.70 months (±10.953) in scoring group 1+ and 90.99 months (+14.64) in group 2+.

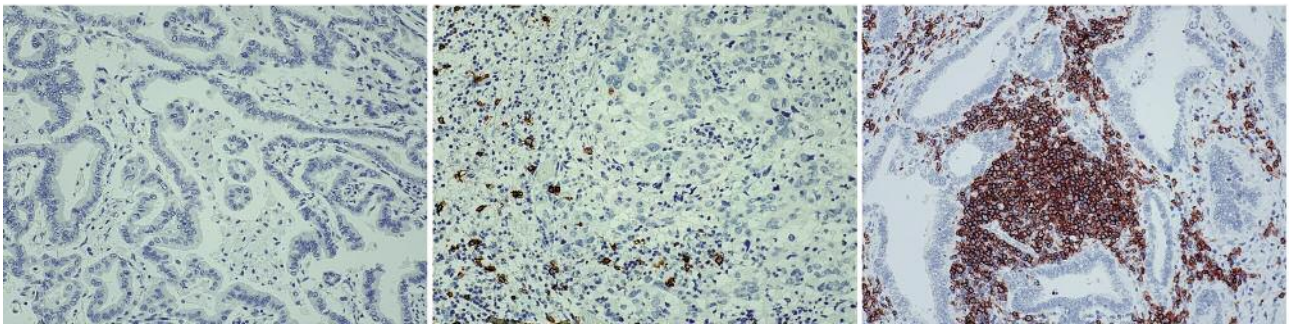

Figure 1. *Assessment of cell density for B-lymphocytes and plasma cells. Left: Score 0 showing no discernible infiltrates. Middle: Score 1 showing scattered cells without dense infiltrates. Right: Score 2 showing dense infiltrates with formation of tertiary lymphatic structures (TLSs). CD20-staining, magnification: ×200.*

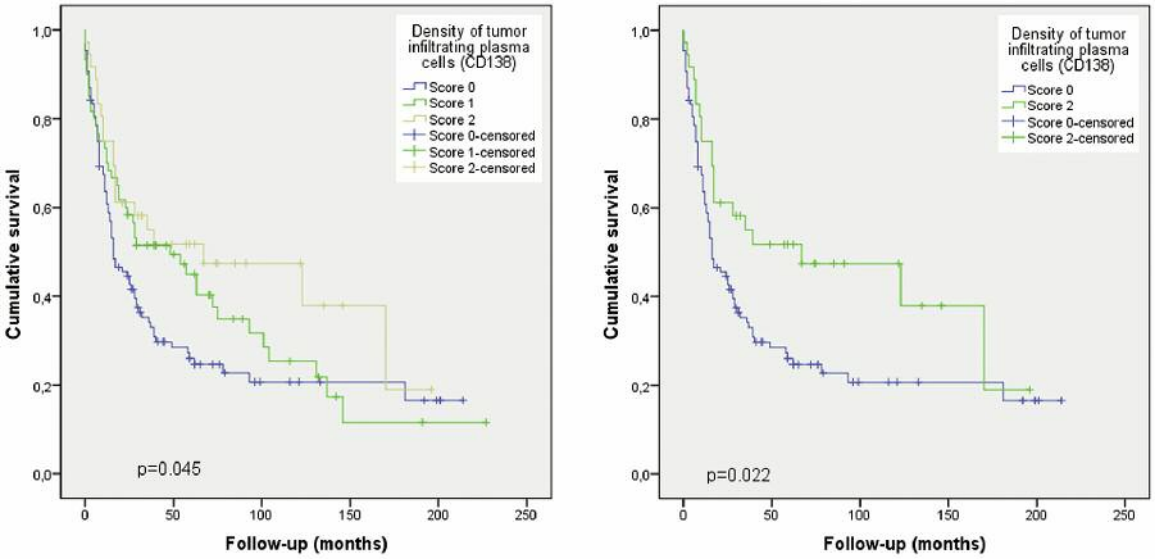

Figure 2. *Kaplan-Meier curves showing survival differences in relation to density of tumor infiltrating plasma cells. Left: Density assessed by scores 0, 1+ and 2+ with a p-value of 0.045. Right: Density assessed exclusively by scores 0 and 2+ with a p-value of 0.022.*

B-cell density in tumor microenvironment. Concerning TIL-Bs, results of analyses showed that there was no significant difference in patients' survival in score groups 0, 1+ and 2+ (p=0.132). However, analysis of patient groups 0 and 2+ also revealed significantly better OS in patients with high B-cell density (p=0.047), as shown in Figure 3.

Survival times in scoring group 0 averaged 67.69 months (±10.579), 63.1 months (±8.505) in score group 1+ and 83.41 months (±15.009) in group 2+.

COX proportional hazard model, adjusted for age, gender and pT-stage revealed that plasma cell density was no independent prognostic factor but showed a trend towards improved survival (hazard ratio (HR)=0.674; 95% confidence interval (CI)=0.369-1.232; p=0.200). Interestingly though, for tumor infiltrating B-lymphocytes, independence could be demonstrated (HR=0.683; 95% CI=0.517-0.901; p=0.007).

Impact of neoadjuvant therapy and high cell density on survival times. Patients with neoadjuvant therapy and high plasma cell density (score 2+, n=10) showed shorter (though not significantly reduced) OS (median survival time=30.15±7.566 months) than patients without neoadjuvant therapy (n=12; median survival=88.13±23.980 months) with a p-value of 0.512. The results were also not significant when taking all three scoring groups into consideration (p=0.715).

Table I. *Clinicopathologic characteristics of adenocarcinoma of the esophagogastric junction according to B-cell (CD20) and plasma cell (CD138) density. Comparison of tumors with no, scattered or dense infiltrates (score 0, 1+ and 2+) with various clinical and pathological features.*

		B-cell scores				Plasma cell scores			
Characteristics		0	1+	2+	*p*-Value	0	1+	2+	*p*-Value
Gender									
	Male	67	70	39	0.664	91	49	31	0.835
	Female	10	14	9		17	11	5	
pT									
(low)									
	pT0	2	2	1	0.712	2	1	2	0.477
	pT1a	3	3	1		3	1	2	
	pT1b	6	10	10		11	9	7	
	pT2	12	17	9		17	10	10	
(high)	pT3	47	44	20		64	31	13	
	pT4a	6	5	5		8	6	2	
	pT4b	1	3	2		3	2	0	
pN									
	pN0	24	36	17	0.773	34	23	18	0.300
	pN1	14	13	6		16	10	7	
	pN2	20	16	12		31	12	4	
	pN3	19	19	12		27	13	7	
LVSI									
	Present	50	41	26	0.091	66	28	19	0.160
	Absent	26	43	22		41	32	17	
Pn invasion									
	Present	22	21	8	0.299	30	14	6	0.377
	Absent	54	63	40		77	46	30	
Grade									
	G1	1	1	0	0.326	2	0	0	0.465
	G2	23	13	12		23	12	12	
	G3	33	45	21		49	32	14	
Distant metastases									
	Present	14	19	13	0.397	26	15	4	0.558
	Absent	36	32	18		44	26	13	
WHO classification									
	Tubular	59	62	33	0.838	75	45	39	0.475
	Poorly cohesive	9	11	6		15	9	2	
	Other	9	11	9		18	6	5	

LVSI, Lymphovascular space invasion; Pn, perineural.

In relation to density of B-lymphocytes, there was no detectable difference in OS for patients with neoadjuvant therapy and high B-cell density (score 2+, n=15) with a median survival of 73.70±12.718 months, while patients without neoadjuvant therapy in scoring group 2 (n=17) showed survival times of 76.84±19.507 months (p=0.411).

Correlation of cell density with most common histologic subtypes. Morphologically, 154 cases (73.3%) were classified as tubular adenocarcinoma, 26 (12.4%) as poorly cohesive carcinoma with signet ring cells and 30 cases (14.3%) as other entities (*i.e.* undifferentiated, mucinous, papillary).

Survival times between tubular adenocarcinoma and poorly cohesive carcinoma did not differ significantly in regard to plasma cell infiltration (mean survival=68.7 *vs*. 56.04 months; p=0.111) or B-cell density (mean survival=71.31 *vs*. 56.04 months; p=0.2).

There were no significant differences between histological subtypes concerning density of plasma cell (p=0.349) or B-cell infiltration (p=0.936).

Interestingly though, the subgroup of poorly cohesive adenocarcinoma showed significantly lower OS rates (n=15; mean survival=16.07±6.452 months) in comparison to tubular adenocarcinomas (n=75; mean survival=62.43±9.916 months) when looking at cases where no plasma cell

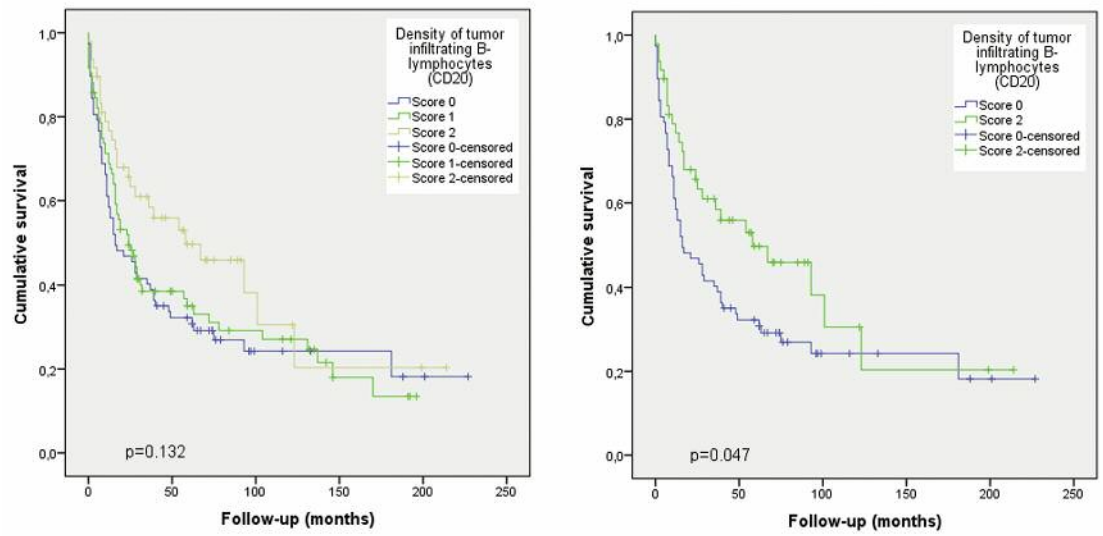

Figure 3. *Kaplan-Meier curves showing survival differences in relation to density of tumor infiltrating B-lymphocytes. Left: Density assessed by scores 0, 1+ and 2+ with a p-value of 0.132. Right: Density assessed exclusively by scores 0 and 2+ with a p-value of 0.047.*

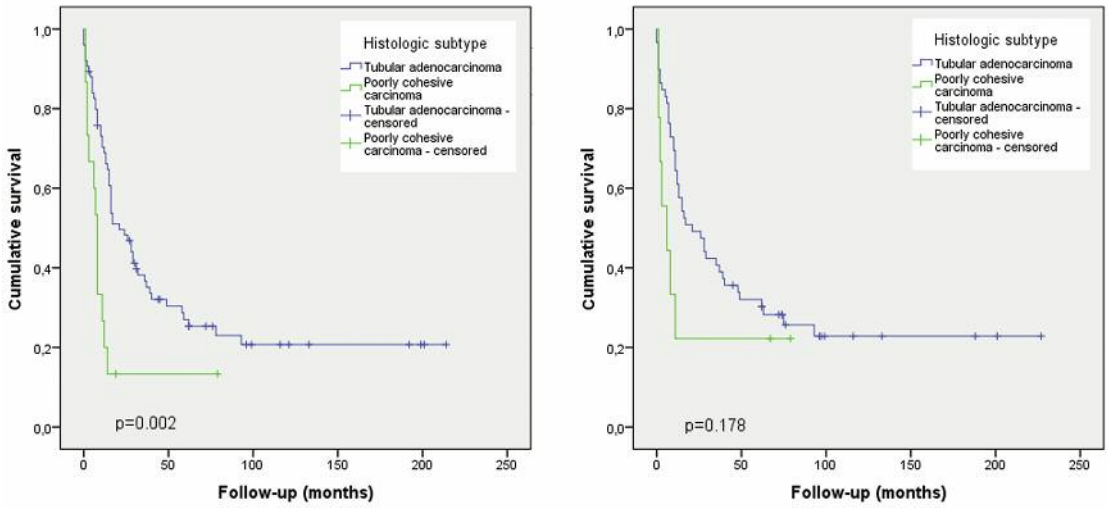

Figure 4. *Kaplan-Meier curves showing survival differences in relation to histologic subtype. Left: Cases with no plasma cell infiltrates (score 0) show significantly better overall survival (OS) if tubular adenocarcinoma is present (p=0.002). Right: Cases without B-lymphocytes (score 0) show no disparity in OS between different histologic subtypes (p=0.178).*

infiltrates (score 0) could be detected (p=0.002). No such correlation could be demonstrated for cases without tumor infiltrating B-cells (p=0.178). Appropriate Kaplan-Meier curves are depicted in Figure 4.

Discussion

In our present study, the relation between density of B-cells and plasma cells in the tumor microenvironment and patients' outcome was investigated. The anti-tumorigenic properties of immune cells (T-, as well as B-lymphocytes) are well-established and high levels of TILs are commonly associated with improved survival in a variety of tumors, including gastric carcinomas (2, 23).

We demonstrated that, in adenocarcinoma of the esophagogastric junction, increased infiltration by B-cells and plasma cells in the stroma indicate prolonged survival, which has not been reported previously. For plasma cells,

this held true for scattered, as well as dense, infiltrates (p=0.045 and p=0.022, respectively), while, for B-cells, the effect was only discernible when so-called TLSs were present (p=0.047). This is in concert with previous findings regarding pancreatic adenocarcinomas where only dense infiltrates and not scattered cells have significant impact on survival times (14), seemingly attributable to a more efficient and active anti-tumorigenic milieu (2). Not only cellular mechanisms mediated through B- and T-lymphocytes but also humoral activity –measured by plasma cell content– seem to have considerable influence on the hosts' inflammatory response to tumor cells. In fact, our results show plasma cells to be yet a stronger indicator of improved survival with even scattered infiltrates showing discernible differences in outcome compared to patients without detectable infiltrates (mean survival=70.70 *vs*. 58.72 months, respectively). However, this could not be confirmed by Cox regression where density of plasma cell infiltration did not constitute an independent prognostic factor (HR=0.674; 95% CI=0.369-1.232; p=0.200). Nevertheless, independence could be demonstrated for B-cell infiltrates with a HR of 0.683 (95% CI=0.517-0.901; p=0.007).

Interestingly, there was no correlation with any of the investigated clinical or pathological parameters on univariate analysis, especially no association with commonly prognosis-related features, such as lymphovascular invasion, lymph node involvement (pN) and depth of invasion (pT) or histologic type. This could partly be attributed to low number of cases in certain subgroups (*i.e.* pT0, pT1a or pT4b) and resulting limitations for statistical analysis. In other groups with higher case numbers, it might be proposed that the prognostic impact of tumor infiltrating lymphocytes is based on mechanisms in the tumor microenvironment that are fundamentally different (and independent) from those that cause survival differences through essentially tumor-related mechanisms (as mentioned above).

There was no significant association between cell density and neoadjuvant therapy (p=0.715 and p=0.411), although survival tended to be shorter in patients with neoadjuvant therapy and high cell densities (especially concerning plasma cells). Therapy itself might probably alter the microenvironment dramatically through increased tumor cell death and raised levels of host immune response. Further studies, preferably evaluating density of tumor-infiltrating cells pre- and post-therapy, could shed more light on this issue.

In conclusion, as high density of tumor-infiltrating plasma cells, as well as B-lymphocytes, correlate with prolonged OS, our results could provide further diagnostic utility in identifying patients with favorable disease course, as well as possible immunotherapeutic treatment strategies for a defined subset of patients. Additionally, investigations of larger cohorts might disclose further insight into the relationship between, *e.g.*, histologic tumor type and density of plasma cells and B-lymphocytes or association with specific clinicopathologic features.

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